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Multiplex PCR for Detection of Exfoliative Toxins, and Toxic Shock Syndrome Toxin 1 Genes in MRSA Strains of Clinical *Staphylococcus aureus* Isolates

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Abstract
This research approaches to investigate toxin gene content of the methicillin resistant *Staphylococcus aureus* strains that are clinically collected from human skin infection cases by performing multiplex polymerase chain reaction (PCR) technique (irrespective of whether the strain produces the toxin or not) and for that a single PCR reaction was employed, two pairs of exfoliative toxin and one pair toxic shock syndrome toxin-1. Amplicon sizes ranged between 93, 226 and 326bp, to facilitate electrophoretic separation. Results of the current study had revealed that the major gene detected was *tst* found in six isolates. Multiple toxin gene combinations were also observed. This work was conducted at several institutions including Tikrit Teaching Hospital's lab; college of science biology department labs/ Tikrit University; and the Biotechnology Research Centre/ Al-Nahrain University.

Key words: *Staphylococcus aureus*, PCR, MRSA, Exfoliative toxin, Toxic shock syndrome toxin-1

Introduction
Exfoliative toxin (ET) produced by *Staphylococcus aureus* strains is the major causative agent of blistering skin disorders, The epidermolytic Exfoliative toxin, capable of causing scalded skin syndrome or bullous impetigo is one of these extracellular proteins that causes a blistering skin disease, staphylococcal scalded skin syndrome (SSSS). It primarily affects neonates and young children. This toxin is involved in some cutaneous infections by targeting desmoglein 1 (Dsg1), a desmosomal cell–cell adhesion molecule. ([1], [3],[4],[2]). Virulent strains of *Staphylococcus aureus* produce two serotypes of exfoliative toxins (ETs), ETA and ETB. Although ETA and ETB have identical biological activity and a degree of genetic similarity, the gene coding for ETA is chromosomal and heat stable whereas the gene coding for ETB is plasmid linked and heat-labile ([1],[5],[6],[7]) ETB is more frequently isolated than ETA in children with generalized Staphylococcal scalded-skin syndrome (SSSS) ([8]). SSSS caused by antibiotic-resistant strains of *Staph. aureus* has recently emerged as an even more serious problem. In SSSS, *Staph. aureus* is present at distant foci such as the pharynx, nose, ear, or conjunctiva, and ET produced by *Staph. aureus* gets into circulation and causes exfoliation at remote sites, whereas in "bullous impetigo", a localized form of SSSS, *Staph. aureus* is present in the lesions (restricted to the sites of infection) ([9],[10],[7]). Staphylococcal Toxic Shock Syndrome toxin (TSST-1) is a Pyrogenic exotoxins that stimulate the release of cytokines and can cause rash, fever, and toxic shock syndrome. This exotoxin is analogous to the pyrogenic toxin produced by Lancefield group A beta-hemolytic streptococci, but is far more deadly. This exotoxin causes toxic shock syndrome and is found in 20% of *Staphylococcus aureaus* isolates. These pyrogenic toxins are called superantigens and bind to the MHC class II molecules on antigen presenting cells (such as macrophages). The toxin-MHC II complex causes a massive T cell response and outpouring of cytokines, resulting in the toxic shock syndrome. Most *Staph. aureus* strains isolated from patients with toxic shock syndrome (TSS), a severe acute illness that rapidly leads to multi-organ system failure, produce a toxin known as toxic shock syndrome toxin-1 (TSST-1). ([12],[13],[14]). Recent studies have suggested that membrane-damaging staphylococcal exotoxins, such as α -toxin, facilitate TSST-1 penetration across vaginal tissue by cytotoxic and proinflammatory disruption of the mucosa ([15]).
The aim of this study was to investigate the presence of genes encoding staphylococcal exfoliative toxins (ETAs, ETBs), and toxic shock syndrome toxin-1 (TSST-1) by multiplex PCR in MRSA isolates. In this paper, we highlighted the factors and parameters that influenced the performance of multiplex PCR to provide detailed description.

Material & Methods
Bacterial Strains
The study employed a total of 35 staphylococcal isolates collected from human skin infections via using superficial swabs while a stab incision (using a sterile needle puncture) was submitted to collect the purulent material in the absence of an open lesion or pus as suggested by dermatologist during the period from February till July 2013. Samples that had exclusively fermented the mannitol of the mannitol salt agar medium and were identified at the species level by the morphological and standard biochemical methods as described by ([16],[17]). However for molecular study only 20 isolates of methicillin resistant *Staph. aureus* (MRSA) provided from Baghdad University, college of science, Biotechnology department were submitted to the PCR reactions. This work was conducted at several institutions (Tikrit Teaching Hospital; Department of microbiology and molecular laboratory at the college

of science; central lab/ Tikrit University; the Biotechnology Research Centre at Al-Nahrain University).

Genomic DNA Extraction

Genomic DNA was extracted from staphylococcal cultures manually via heat shock/boiled-cell method that involved boiling and freeze-thawing processes as described by ([18], [19]) The extracted DNA yield was used as a template for amplification using primers and thermal profiles presented elsewhere.

DNA purity and concentration

The concentration and purity of total DNA isolates in the samples were measured spectrophotometrically at wavelengths of A260 and A280. It was performed in a Nano Drop machine (Thermo Scientific).

DNA amplification

All the processes of DNA amplification were performed with the use of *mecA* and *femA* genes for the confirmation of MRSA stains following the procedure published by ([20]). The MRSA strains were exclusively used to proceed for the multiplex PCR assay to detect the toxin gene content.

Multiplex Primer Conditions & Reaction

The nucleotide sequences of the primers used in this study for the multiplex PCR and their respective amplified products are listed in the following table:

Table (1) Nucleotide sequences, gene locations, and anticipated sizes of PCR products for the MRSA gene-specific oligonucleotide primers as described by [21].

Gene	Primer	Oligonucleotide sequence	Size of amplified product (bp)
eta	GETAR-1 GETAR-2	GCAGGTGTTGATTTAGCATT AGATGTCCCTATTTTTGCTG	93
etb	GETBR-1 GETBR-2	ACAAGCAAAAGAATACAGCG GTTTTTGGCTGCTTCTCTTG	226
tst	GTSSTR-1 GTSSTR-2	ACCCCTGTTCCCTTATCATC TTTTCAGTATTTGTAACGCC	326

A multiplex PCR amplification was employed using pairs of exfoliative toxins and tss-1 primers. The reaction mixture were prepared according to the instruction of Acuu Power PCR PreMix from (BiONEER, Korea), with slight modifications to the procedure written by ([21]).The primers, used were at 10 pmol for *eta*, *etb* and *tst*. The volume of this mix was adjusted to 50 µL with sterile water. DNA amplification was carried out in a Labnet thermocycler with the following thermal cycling profile: an initial denaturation at 94°C for 5 min was followed by 35 cycles of amplification (denaturation at 94°C for 2 min, annealing at 57°C for 2 min, and extension at 72°C for 1 min), ending with a final extension at 72°C for 7 min. PCR products (10 µl) were obsreved in a 2% agarose gel in 1x TBE buffer 80 V for 2 hours or approximately 200 min to separate the different amplification products efficiently. The gel was stained with ethidium bromide and photographed using gel documentation system ([22], [23]). The molecular weights of bands were estimated by using standard molecular weight marker.

Figure (1) Electrophoresis gel for genomic DNA of *Staph. aureus* isolates by electrophoresis on 1% agarose gel stained with ethidium bromide

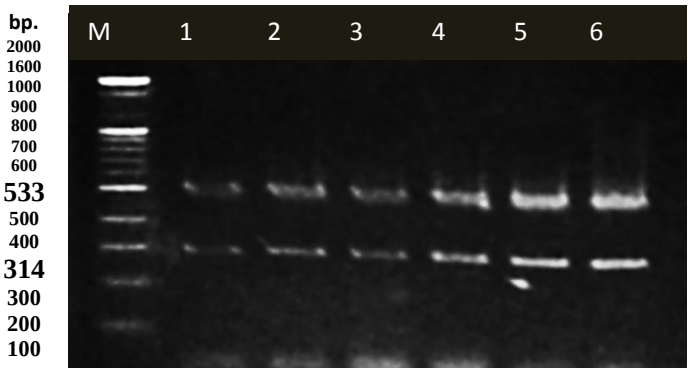

Figure (2) Ethidium bromide-stained agarose gel (2%) electrophoresis of DNA fragments generated by duplex PCR amplification for detection of (MRSA). Lane (M): 100bp DNA ladder (Bioneer), lanes (1-6): PCR product of *femA* gene (314bp) and *mecA* gene (533bp).

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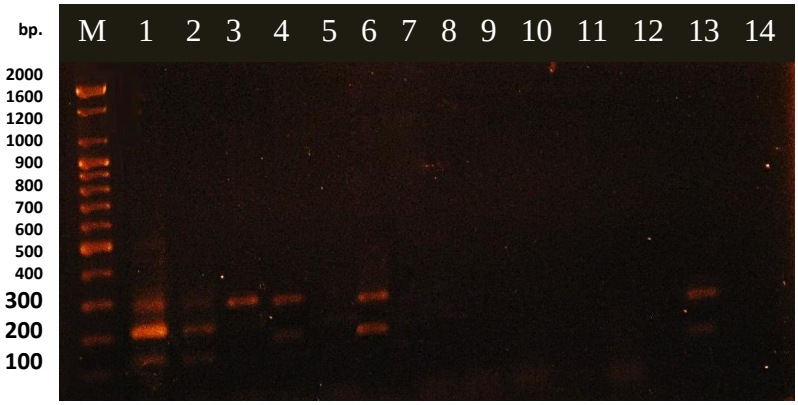

Figure (3). 2% Agarose gel electrophoresis of the Set B multiplex PCR amplification products (*eta* 93bp, *etb* 226bp, *tst* 326bp) obtained from analysis strains. MW = molecular weight marker 100 bp ladder.

General Considerations for Multiplex PCR

This research focuses on multiplex systems in which each primer pair targets a single locus, however there are considerations that should be taken when handling with this assay:

- Concerning with the results, extra faint bands were detected with the primers, possibly, corresponding to nonspecific binding, primarily because of the formation of primer dimers. These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension Thus, the optimization of multiplex PCR should aim to minimize or reduce such nonspecific interactions.
- For a better separation of product multiplex PCR we suggest to separate the product size of the genes *eta*, *etb*, *tst* in a better way by increasing concentration of agarose to 3% otherwise replace polyacrylamide instead of agarose in the gel for the electrophoresis step.

PCR bands, differing from each other by 30-40 bp in length could be conveniently separated on 3% gels. Overnight separation of products at lower voltage gradients notably decreased the sharpness of individual PCR bands, especially when the products were smaller than 400-500 bp ([28]).

- Amount of primer. Initially, equimolar primer concentrations of 10 picomole for each were used in the multiplex PCR, but there was uneven amplification, however the yield bands with the10 pmol. were better than the first result with 100 and 50 pmol. [28] had advised that overcoming this problem require changing the proportions of various primers in the reaction, with an increase in the amount of primers for the "weak" loci and a decrease in the amount for the "strong" loci but they also had mentioned that when the multiplex reaction is performed for the first time, it is useful to add the primers in equimolar amounts.
- At DNA template quantities between 30 and 500 ng/25mL reaction, mixture showed no significant differences; however, below 30 ng the amount of some of the products decreased ([28]).
- Factors preventing optimal annealing rates include poorly designed primers and suboptimal buffer constituents and annealing temperature. The extension rate of specific primer-target hybrids depends on the activity of the enzyme, availability of essential components such as deoxyribonucleoside triphosphates (dNTPs), and the nature of the target DNA ([25]).

Results and Discussion

Genomic DNA obtained from the manual boiling method was used for molecular investigation. A spectrophotometric method based on measuring the amount of ultraviolet (UV) irradiation that is absorbed by the bases was performed in order to assess the purity of the genomic DNA samples. The boiling method is simple and rapid involving fewer steps, capable of handling large number of samples in a short span of time, does not need any special equipment and chemical (except for a centrifuge) and does not contain any hazardous chemical in the extraction buffer ([30]). Specific polymerase chain reaction had been made to identify the MRSA strain by amplification of *mec*A gene fragments derived from isolated clinical specimens directly with the detection of a certain marker which is specific for *Staph. aureus* that is needed to distinguish MRSA from other species of staphylococci. In this research, we also report the development of polymerase chain reaction (PCR) procedures which will rapidly and specifically detect genes for staphylococcal TSST-1, and exfoliative toxins A and B in strains of MRSA associated with human disease by multiplex PCR assay (gene screening) as an alternative to the conventional biological assays, which depend on detectable amounts of toxins produced. Multiplex PCR included three genes. Not all isolate samples were listed in the same figure however, Details of this assay are illustrated next in the following table.

Table (2) Distribution of toxin genes in the selected MRSA isolates

Isolate number	Gene combination		
	eta	*etb*	*tst*
MRSA1	+	+	+
MRSA2	+	+	+
MRSA3	-	-	-
MRSA4	-	+	+
MRSA5	-	-	-
MRSA6	-	+	+
MRSA7	-	-	-
MRSA8	-	-	-
MRSA9	-	-	-
MRSA10	-	-	-
MRSA11	-	-	-
MRSA12	-	-	-
MRSA13	-	+	+
MRSA14	-	-	-
MRSA15	-	-	-
MRSA16	-	-	-
MRSA17	-	-	-
MRSA18	-	-	+
MRSA19	-	-	-
MRSA20	-	-	-

Using this PCR assay we found that among the MRSA strains obtained (n=20), our results showed that most of MRSA samples harbored at least one virulence gene. Multiple toxin gene combinations were also observed. The major gene detected was *tst* in six isolates, each of the six isolates containing the *tst* gene harbored at least one classical ET gene; four isolates possessing the *tst-etb* gene combination, and two also had the eta combined with *tst* gene.

Our findings of the toxin gene content of MRSA isolates agreed with Moore and Lindsay (2001) who reported that *Staph. aureus* strains often carry in their genomes virulence genes that are not found in all strains and that may be carried on discrete genetic elements. In conclusion, the results of the present study indicate that the most common associated toxin among our clinical isolates is the toxic shock syndrome toxin-1. This research focuses on multiplex systems in which each primer pair targets a single locus, unlike RAPD PCR reactions, which amplify multiple loci with a single primer. From these results, and from our own experience with multiplex PCR, it has become evident that single template PCR Reaction is ideal for conserving costly polymerase and templates in short supply and can be applied in Pathogen Identification.

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تفاعل البلمرة المتسلسل المتعدد (Multiplex-PCR) لتحديد جينات سموم متلازمتي تقشر الجلد والصدمة السمية نوع–1 في سلالات من بكتيريا المكورات العنقودية الذهبية المعزولة سريرياً

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الملخص

تهدف الدراسة الى التحري عن المحتوى الجيني من الذيفانات في سلالات بكتيريا المكورات العنقودية الذهبية المقاومة لمضاد المثيسيلين المعزولة سريرياً من أشخاص مصابين باصابات جلدية باستخدام تقنيه تفاعل البلمرة المتعدد (بغض النظر اذا كانت السلاسة منتجه للذوفان او لا) حيث خضعت للدراسة على المستوى الجزيئي بعد أن أُستُخلص المحتوى الوراثي بإستخدام طريقة الغليان والتي اعطت نتيجة تراكيز للDNA تراوحت مابين 150 الى 400 نانوغرام/مل وبنسبة نقاوه نوعيه 1.5 .تتضمن تقنيه البلمرة المتعدد تفاعل واحد أُستخِدم فيها زوج من الجينات التي تُشّفر لذوفان تقشِر الجلد مع جين واحد يشّفرلذوفان متلازمة الصدمة السمية نوع–1. تراوح حجم القطع الناتجة من التفاعل 93,226 و 326 زوج قاعدي لضمان فصلهم في مرحلة الترحيل الكهربائي وقد أظهرت نتائج دراستنا بأن أكثر جين تم تحديده هو الجين *tst* حيث لوحظ تواجده بشكل مفرد او مجتمعا مع الجينين الاخرين. تم إجراء هذا البحث في عدة مؤسسات من ضمنها مختبر مستشفى تكريت التعليمي ومختبرات كلية العلوم/ جامعة تكريت و مركز الأبحاث والتقانات الحيوية/ جامعة النهرين.